THE PRODUCTION OF FLUORESCENT PIGMENT BY BACTERIA.

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A CONSIDERABLE number of different "species" of bacteria have been described as endowed with the property of forming a blue-green fluorescent pigment in suitable media. I append a list, perhaps not complete, of the various "fluorescent bacteria" already discovered.

It is not at all likely that the fifty names bestowed upon these cultures represent as many totally distinct microorganisms, and in some cases it is practically certain that the same bacterium masquerades under several different titles.

The experiments recorded in the present paper embody a series of attempts to discover the conditions under which fluorescence is produced, and especially the nature and amount of the chemical substances essential to the formation of the fluorescing body.

I have chosen for this purpose six different cultures.[1] Four of these do not liquefy gelatin and were sent to me from Kral's Laboratorium under the names *B. fluorescens albus*, *B. fluorescens tenuis*, *B. fluorescens mesentericus*, and *B. fluorescens putridus*. The two others liquefy gelatin ; one of them was sent by Kral with the name *B. viridans*, and the second was isolated by me from the water of Lake Michigan and identified as *B. fluorescens liquefaciens* (description by Kruse in Flügge's Die Mikroorganismen **2** : 292).

1. B. FL. ALBUS.— A bacillus was first described under this name by Zimmermann who found it in the Döbeln water supply.[2] The culture bear-

[1] I have also made some experiments with *B. pyocyaneus*, but, owing to the complication due to the formation of at least two pigments by this bacillus, I am led to reserve my statements on this head for a subsequent communication.

[2] O. E. R. Zimmerman, Die Bakterien unserer Trink- und Nutzwässer 18. Chemnitz, 1890.

ing this name which I have employed agrees closely with Zimmermann's description. The bacilli are small (0.6 μ by 1–2 μ) with rounded ends, and occur both singly and in short chains; they are actively motile. Upon gelatin plates the colonies spread out into a thallus-like expansion and the surrounding medium assumes first a blue-green and later a grass-green tint. The growth on agar is elevated and translucent white, and a green fluorescence is imparted to the medium. The growth on potato is luxuriant, and has a rich chocolate-brown color. Milk is not coagulated, no indol is formed, and nitrate is not reduced. In the fermentation-tube no gas is produced, but the broth is rendered strongly acid. At 37.5° C. a good growth takes place upon agar, but no pigment is produced at this temperature. A perceptible amount of pigment is formed at 33° C.

2. B. FL. TENUIS.— Described by Zimmermann[3] as a distinct species. Found also by Dittrich[4] and regarded by him likewise as a distinct species. Kruse,[5] however, considers this organism as merely a variety of *B. fluorescens non-liquefaciens*. My own culture bearing the name of *B. fluorescens tenuis* agrees very closely with *B. fl. albus*. The chief points of difference are (*a*) its failure to grow at all at 37.5° C.; (*b*) its ability to produce a small amount of indol in sugar-free broth.

The fine, delicate leaf-like growth in streak-cultures, upon which Zimmermann chiefly bases his separation of this "species" from *B. fl. albus*, was noticed sometimes in my culture, but was not constant.

3. B. FL. MESENTERICUS.— Described by Tartaroff.[6] I have not been able to see the original description. The culture sent me under this name resembles *B. fl. albus* very closely. There are slight but inconstant differences in the growth of the two cultures upon gelatin, agar, and potato. The growth at 37.5° C. is much less profuse than with *B. fl. albus* and no pigment is formed when the culture is kept at 33° C. Milk is slightly digested and acquires a feebly acid reaction.

4. B. FL. PUTRIDUS.— Described in Flügge's Die Mikroorganismen, 2d edition, p. 288; cf. also the 3d edition, **2**: 292. My own culture bearing this name agrees in most respects with *B. fl. albus*. The chief differences are (*a*) much scantier growth at 37.5° C.; (*b*) less rapid growth on all media; (*c*) a dry, thin growth on potato, more restricted and duller than that of *B. fl. albus*; (*d*) a much more decided green tinge imparted to milk.

These differences, although slight, have been constant during some ten months of observation. I have not been able to distinguish any difference between the character or intensity of the odor given off by this species and that observed in the three cultures previously described.

[3] *Op. cit.* p. 16.

[4] Verhandl. d. naturhistor. Vereins zu Heidelberg N. F. **5**: 536. 1897.

[5] Flügge, Die Mikroorganismen **2**: 293.

[6] Die Dorpater Wasserbakterien. Inaug. Diss., 1891.

5. B. VIRIDANS.— A microorganism was first described under this name by Symmers.[7] Zimmermann[8] also described a form found in polluted water which he pronounces wholly identical with a culture of *B. viridans* (Symmers), obtained from Kral. My own culture bearing this name was sent me from Kral's collection.

The bacilli vary considerably in dimensions when grown on different media. They are short and quite slender in broth cultures, longer and plumper on potato. On the latter medium they average about 0.8–0.9μ by 2.5–3μ. They occur usually singly or in pairs when grown in broth, and are non-motile. They stain with the ordinary aniline dyes, but not so readily as with carbol-fuchsin or Löffler's methylene blue. They do not retain the stain by Gram's method. Gelatin is liquefied very rapidly, the growth in a stab-culture occurring at the upper part of the inoculation line and giving rise to a saucer-shaped depression. The growth in agar streak-culture is gray, thin, fairly spreading and with serrated edges. The medium is speedily colored a fine blue-green which in seven days has changed to a dark Nile-green. The growth on potato is luxuriant, at first dry and of a tan-color, but later becoming dark and slimy. Milk is curdled with acid reaction, and the whey assumes a decided green color. No indol is formed, and nitrate is not reduced. In the fermentation tube no gas is produced in glucose bouillon but the medium becomes strongly acid. At 37.5° C. a very scanty growth occurs, but no pigment is produced.

Zimmermann[9] classifies this organism as one that produces green, but not fluorescent pigment. I have not been able to remark the existence of any such difference between my culture of *B. viridans* and the other organisms I have studied; fluorescence is manifest in all.

6. B. FL. LIQUEFACIENS.— A germ isolated by me from the water of Lake Michigan. I have given it the above name, since the culture agrees with the description of the species by Kruse.[10] The chief differences between this culture and that of *B. viridans* are the following: (*a*) no growth at all occurs at 37.5° C.; (*b*) growth in gelatin stab-cultures is more rapid than that of *B. viridans* under the same conditions, but pigment is less abundantly produced; (*c*) the bacilli are actively motile; (*d*) growth on potato is from the outset moist and slimy; (*e*) nitrate is readily reduced to nitrite.

It is evident from these descriptions that the existing differences between some of the cultures I have employed are hardly to be regarded as specific, whatever may have been the case with the type microorganisms when first isolated. I shall for conve-

[7] British Medical Journal **2**: 1252. 1891; cf. also Brit. Med. Journ. **1**: 113. 1893.
[8] *Op. cit.* **2**: 22. [9] *Op. cit.* **2**: 30.
[10] Flügge's Die Mikroorganismen, *loc. cit.*

nience, however, refer to the cultures by the names they bear just as if they were so many distinct "species."

The methods employed have been selected with a view to obtaining constancy and uniformity of conditions. Owing to the familiar fact that the different commercial peptones vary widely in chemical composition and nutritive value, and since Gessard[11] and Lepierre[12] have definitely proved that the production of fluorescence by certain bacteria is profoundly affected by the nature of the peptone used, I have carried out my investigations with the aid of simple solutions of chemical compounds whose molecular composition and arrangement are more accurately known. The nitrogenous basis of these nutrient media has consisted of asparagin or ammonium salts, and to these have been added other substances according to the nature of the experiment. The utmost care has been taken to obtain chemicals of strict purity, since a mere trace of foreign substance is in some cases sufficient to vitiate the result.

In all of my solutions I have employed water redistilled in glass. The various flasks and test-tubes used in my experiments have been thoroughly cleansed with chromic acid cleaning mixture which has been very carefully washed out and the vessels finally rinsed in distilled water. A suitable quantity (usually 7–8^{cc}) of the nutrient solution has been placed in thin-walled test-tubes and sterilized in the steam-bath by the discontinuous method. Prolonged heating and the use of the autoclave have been avoided as they have been found to affect the constitution of some of the media.

Inoculation of the media has usually been made from vigorous agar growths about three to five days old, but I have also often used for this purpose fluid media in which abundant production of pigment had occurred, *e. g.*, an asparagin-phosphate-sulfate solution, but without noting any difference in the outcome.

The cultures have always been kept in the dark and at the room-temperature, 18–20° C., except when specified otherwise.

[11] Ann. de l'Inst. Past. **6**: 801. 1892.

[12] Ann. de l'Inst. Past. **9**: 643. 1895.

The most extended contribution to our knowledge of the fluorescent bacteria, with the exception of Gessard's classic papers upon *B. pyocyaneus*, is contained in a paper by Thumm.[13] My own observations conflict with Thumm's at some points, while the conclusions I am compelled to draw from my results are, in important respects, so at variance with his that I do not hesitate to reopen the whole question and to present the record of my experiments in some detail.

INFLUENCE OF THE CHEMICAL COMPOSITION OF THE MEDIUM.

In 0.2 per cent. pure asparagin solutions a very slight turbidity occurs, but no trace of color. If 0.1 or 0.5 per cent. of chlorid (sodium, calcium, magnesium, or potassium) be added to the asparagin solution, the turbidity is slightly increased, but no pigment is formed. Sulfates (sodium, magnesium, or potassium) in the same proportion aid the growth more than chlorids, but no color appears. Pure phosphates (sodium, magnesium, or potassium) are yet more favorable to growth, but fluorescent pigment still fails to develop. I must at this point lay stress upon the importance of obtaining chemically pure phosphates. A mere trace of sulfate in the presence of phosphate, as I shall show presently, is sufficient to lead to the production of pigment. I have found that many samples of "C. P." phosphate obtained from reliable manufacturers contain a quantity of sulfate sufficient to vitiate the results, and I have consequently been compelled to prepare pure phosphates by recrystallization or by thorough washing.[14]

If the various species be grown in a solution of 0.2 per cent. asparagin, 0.1–0.5 per cent. sodium (or magnesium) chlorid, and 0.1 per cent. neutral sodium phosphate, the resulting turbidity is but little, if at all, greater than in the asparagin-phosphate solution alone. If, however, 0.1 per cent of sulfate (sodium,

[13] Beiträge zur Biologie der fluorescierenden Bakterien. Arb. d. Bakteriol. Inst. d. grossh. Hochschule zu Karlsruhe, 1895.

[14] I am indebted to my friend Professor Stieglitz for many suggestions regarding the preparation and testing of pure salts.

magnesium, or potassium) be added to the asparagin-phosphate solution, abundant multiplication takes place together with a rich development of the fluorescent pigment.

To determine the amount of sulfate necessary for the production of the pigment, solutions were prepared containing 0.2 per cent. asparagin; 0.1 per cent. neutral sodium phosphate; and respectively 0.1, 0.01, 0.001, 0.0001, and 0.00001 per cent. magnesium sulfate. All of the species except *B. fl. putridus* produced pigment in the presence of 0.01 per cent. and 0.001 per cent. sulfate nearly as well as when 0.1 per cent. was used; with the smaller amount of sulfate, the development of pigment was slower, but the final result almost as intense. With 0.0001 per cent. of sulfate a good development of color was brought about by all five species. In the presence of 0.00001 per cent. of sulfate *B. viridans* alone showed any well-defined development of pigment. There was the merest trace of color in *B. fl. mesentericus*, and the others were entirely without a tinge.

The nature of the base associated with the phosphorus and sulfur appears to be a matter of complete indifference. Sodium, potassium, and magnesium salts gave similar results in whatever way they were combined. Even if ammonium phosphate and ammonium sulfate be used together, fluorescence appears, but it is somewhat less intense than in the presence of one of the bases mentioned above.

To determine the amount of phosphate necessary for the formation of pigment, solutions of 0.2 per cent. asparagin, 0.1 per cent. magnesium sulfate, and varying quantities of neutral sodium phosphate were inoculated in the usual manner. In the asparagin-sulfate solution containing 0.001 per cent. of the phosphate, good color was developed by all the species, being deepest in the cultures of *B. viridans* and *B. fl. mesentericus*. In the same solution containing 0.0001 per cent. of phosphate no pigment was developed by any of the species; the turbidity was, however, slightly more pronounced than in the control tubes of simple asparagin-sulfate solution.

These organisms are able to produce the fluorescent pigment

for a series of generations in such simple media as I have used. In a solution of 0.2 per cent. asparagin, 0.1 per cent. sodium phosphate, and 0.0001 per cent. magnesium sulfate, *B. fl. albus* is able to develop a fairly intense color. If from a culture in this medium a second tube of the same solution is infected, and the procedure repeated at intervals of three days, neither attenuation nor exaltation of the fluorescigenic power can be noticed after thirteen successive transfers. The same statement holds true of the action of *B. fl. putridus* in a solution containing 0.25 per cent. ammonium lactate, 0.1 per cent. sodium phosphate, and 0.1 per cent. magnesium sulfate.

The solutions of the ammonium salts of the organic acids, with the exception of the urate, contained respectively 0.5 per cent. of the ammonium salt and 0.1 per cent. each of neutral sodium phosphate and magnesium sulfate. Ammonium *succinate*, *lactate*, and *citrate* all proved to be substances well adapted for the production of the fluorescent pigment. The color appears more speedily in the succinate and lactate, and with most species becomes also more intense than in the citrate.

In the *succinate* solution all of the species but one developed pigment quickly and intensely. The color developed very tardily in the culture of *B. fl. putridus*, and never became intense. In ammonium *lactate* solution, a fine color developed within two days in the cultures of *B. fl. albus*, *B. viridans*, *B. fl. tenuis*, and *B. fl. mesentericus; B. fl. liquefaciens* was from ten to twelve hours behind the others at this stage, but eventually produced a very deep color; *B. fl. putridus* showed a much fainter tinge of color after ten days, and the color never became pronounced.

The ammonium *citrate* solution did not, on the whole, lend itself to the production of pigment quite as readily as the lactate and succinate. *B. fl. albus*, *B. fl. tenuis*, and *B. fl. mesentericus*, indeed, seemed to flourish and produce pigment nearly as well as in lactate or succinate, but *B. viridans* and *B. liquefaciens* showed a decidedly less rapid growth, while *B. fl. putridus* caused only a very slight turbidity and developed no trace of color.

Ammonium *tartrate* solution afforded one of the most interesting examples of a difference between the cultures employed. *B. fl. albus* produced a rich coloration of the medium within the space of twenty-four hours. On the tenth day *B. fl. tenuis* likewise showed a slight tinge of color, and this slowly but steadily deepened, until, on the twenty-fifth day after inoculation, the color was as decided as in the tubes of *B. fl. albus*. None of the other species showed the slightest development of pigment, although a marked turbidity manifested itself in the tube inoculated with *B. fl. liquefaciens*, and a perceptible cloudiness appeared in the culture of *B. fl. mesentericus* and *B. fl. putridus*. The difference in the behavior of the several species in this solution was so striking that I repeated the experiment several times, always, however, with the same outcome. It is interesting to note in this connection that Thumm[15] states that the only species (among *B. fl. albus*, *tenuis*, *erythrosporus*, *putridus*, and *viridans*) that was able to produce fluorescent pigment in a tartrate solution (ammonium tartrate 0.5 per cent., potassium phosphate 0.05 per cent., magnesium sulfate 0.01 per cent., calcium chlorid 0.005 per cent.) was the one bearing the name of *B. fl. albus*. Thumm gives no description whatever of the various "species" that he used, but they were probably derived from the same sources as my own cultures. Thumm's culture of "*B. fl. tenuis*" is recorded (p. 84) as showing "slight turbidity: no production of pigment" in the tartrate solution, while my culture designated by the same name is able to form a notable quantity of pigment, although more tardily than *B. fl. albus*. In ammonium *urate* solution (urate 0.05 per cent.,[16] sodium phosphate 0.1 per cent., magnesium sulfate 0.1 per cent.), *B. fl. liquefaciens* produces pigment more rapidly than the other species, but at the end of seven days *B. fl. mesentericus* and *B. fl. tenuis* rival it in intensity, and after eighteen days growth *B. fl. albus* shows quite as deep a color as the species already named. There is at this time the

[15] *Op. cit.* p. 84.

[16] On account of the comparatively slight solubility of the urate a smaller quantity was used than of the more soluble ammonium salts.

merest dash of color in the tube of *B. viridans*, and none at all in that of *B. fl. putridus*.

Ammonium *acetate* solution gave on the first trial a slight turbidity with *B. viridans*, but no sign of growth with any of the other species. The acetate solution was found, however, to have quite a decided acid reaction, and on rendering it slightly alkaline with ammonia, all the species except *B. fl. putridus* became able to produce a very considerable quantity of pigment.

Ammonium *oxalate* solution is not adapted to the production of pigment although capable of supporting growth. *B. fl. albus* and *B. fl. tenuis* cause decided turbidity, but never develop any trace of fluorescent pigment even when alkali is added to the medium. The other species show varying degrees of cloudiness. If a more dilute solution be employed (0.05 per cent. oxalate), a faint tinge of color appears in the culture of *B. fl. liquefaciens*, and the merest suggestion of fluorescence is shown in the tubes of *B. fl. albus*, *B. fl. tenuis* and *B. fl. mesentericus*.

In ammonium *formate* solution the conditions for growth and pigment production are still less favorable than in oxalate; in the ordinary solution the tubes remain perfectly clear. If the solution be made slightly alkaline, however, a faint tinge of color appears in the culture of *B. fl. liquefaciens*, but the other species, while producing a slight turbidity, never form pigment.

INFLUENCE OF CONCENTRATION OF THE MEDIUM.

Considerable influence upon the production of pigment is exerted by the degree of concentration of the medium. In Uschinsky's solution,[17] which consists of glycerin 30–40^{gm}, sodium chlorid 5–7^{gm}, calcium chlorid 0.1^{gm}, magnesium sulfate 0.2–0.4^{gm}, di-potassium phosphate 2–2.5^{gm}, ammonium lactate 6–7^{gm}, sodium aspartate 3–4^{gm}, dissolved in 1000^{cc} of water, some of the species, notably *B. fl. albus* and *B. fl. mesentericus*, grow luxuriantly and produce a goodly quantity of pigment, but the intensity of color never reaches as high a point as in some of the simpler solutions, and the color assumes much earlier the

[17] Centralbl. f. Bakt. 14:316. 1893.

yellow tinge which overtakes all old cultures. Some of the species, as for example *B. fl. putridus*, are unable to grow in Uschinsky's medium, and the tubes, although inoculated from a young active culture, remain perfectly clear. There is some lack of uniformity in regard to the behavior in this medium. *B. viridans* and *B. fl. tenuis*, for example, will sometimes multiply and produce considerable turbidity without forming pigment, and at others, under apparently identical conditions, will refuse to grow at all. The presence of glycerin is one of the unfavorable factors. If the medium be diluted 1:5 or 1:10, all of the species are able to grow and produce pigment.

A medium somewhat similar to Uschinsky's, but simpler, was compounded by Fränkel.[18] This consisted, in its final modification, of sodium chlorid 0.5^{gm}, ammonium lactate 6^{gm}, asparagin 4^{gm}, neutral sodium phosphate 2^{gm}, dissolved in 1000^{cc} of water. The solution was either amphoteric or slightly acid, and was made slightly alkaline "with a small quantity of caustic soda." In my early experiments all the species grew quite luxuriantly in this medium, but never produced any fluorescence, although the old cultures usually had a yellow tinge. The reason for this failure to produce pigment might at first be supposed to lie in the absence of sulfate, especially since color is developed when sulfate is added to Fränkel's medium. But that this is not the whole explanation is shown by the fact that, if the original medium was diluted 1:10, a very fair development of pigment took place. Investigation showed that some of the ingredients of the solution were not perfectly pure, but contained a trace of sulfate. The quantity of sulfate present, however, was not sufficient to permit of pigment production in the strong solution, although one-tenth of the same quantity was sufficient to permit of pigment production in a less concentrated medium. If Fränkel's medium be prepared with perfectly pure chemicals no pigment production occurs even when the solution is diluted.

The effect of concentration is well shown also in experiments made with solutions of high phosphate content. In solutions of

[18] Hyg. Rundschau 4:700.

0.2 per cent. asparagin, 0.0001 per cent. sodium sulfate, and, respectively, 1, 0.5, 0.2, 0.1 per cent. neutral sodium phosphate, *B. fl. albus* develops pigment most rapidly and intensely in the 0.1 per cent. phosphate solution. *B. fl. putridus* and *B. fl. tenuis*, on the other hand, develop pigment soonest in the 1 per cent. phosphate solution, and the former species does not develop any at all in the solutions with slighter phosphate content. As a rule, however, especially in solutions containing a larger amount of sulfate, a phosphate content as high as 1 per cent. interferes with the production of fluorescent pigment and the culture generally assumes a muddy yellow tint. The same thing occurs if a considerable quantity of ammonium salt be employed. *B. fl. albus*, for example, in a solution of 2 per cent. of ammonium tartrate and the usual quantities of phosphate and sulfate, imparts less of a fluorescent tinge and more of a yellow color to the medium, than with the ordinary 0.5 per cent. tartrate solution. This fact is of interest in connection with Lepierre's conclusions,[19] which are based upon experiments made with 2 per cent. solutions of ammonium salts. The high acidity of such strong solutions as he employed, however, was probably an even greater factor in the vitiation of his results than the simple concentration of the medium.

INFLUENCE OF LIGHT UPON PIGMENT PRODUCTION.

Pigment production is perceptibly affected by light. I have made no experiments with regard to the action of direct sunlight, but have confined myself to a comparison of cultures kept in the dark with others kept in diffuse daylight. The latter cultures were kept on a desk near the windows of a room into which no direct sunlight, but only north light entered; the former were kept in a locker in the same room and as nearly as possible at the same temperature, although variations of from three to four degrees were occasionally observed. These slight temperature variations were, however, sometimes on one side, sometimes on the other, and I have seen no reason to infer that the course of

[19] Ann. de l'Inst. Past. **9**:643. 1895.

pigment production is materially influenced by such slight fluctuations. It has invariably happened in these experiments that the cultures kept in diffuse daylight show a distinctly fainter color than the control tubes kept in the dark. In several instances fluorescence failed to appear in cultures grown in the light. This is true, for example, of a culture of *B. fl. albus* in a solution of 0.2 per cent. asparagin, 0.1 per cent. sodium phosphate, 0.0001 per cent. magnesium sulfate. In other solutions too, in which the amount of pigment produced is at best not great, a similar complete inhibition of the fluorescigenic power can be noticed. Thumm (*l. c.*) does not state whether the cultures in his experiments were kept in the dark or in the light, and it may be suggested that if the latter was the case some of his negative results (as, for example, that with *B. fl. tenuis* in ammonium tartrate solution) might be accounted for.

The question whether in such cases the light acts upon the pigment or upon the metabolic activity of the bacteria is a difficult one to come at. The turbidity of cultures kept in the dark and in the light seems to be about the same. If cultures in which abundant pigment production has taken place be transferred to the light (this was done with a set of cultures in ammonium succinate solution), a very slight fading out can be noticed at the end of two weeks. The fading is accelerated by placing the tubes on the window-sill (north light), but even after the expiration of three weeks no very extensive divergence can be noted when the tubes are compared with the control tubes which have remained all the while in the dark. The cultures of some species, however, show a greater tendency to fade than others; the pigment produced by *B. fl. albus* fades out quite readily, while that formed by *B. fl. tenuis* retains its brightness with comparative persistence. The fading out consists in the gradual yellowing of the pigment, which continues until all trace of green is eliminated; cultures kept in the dark show the same change, but it takes place, as I have indicated, more slowly. The change is probably one of oxidation, and the acceleration of the process in the light is precisely what might be anticipated.

The addition of an oxidizing agent, as dilute potassium permanganate, has a similar etiolating effect upon the pigment.

INFLUENCE OF THE REACTION OF THE MEDIUM.

The presence of acid checks the production of pigment Even in slightly acid solutions where considerable multiplication may occur, pigment is not formed. The interference of the acid seems to be rather with the metabolic activities of the bacteria than with the pigment, as the following facts indicate. The pigment when it is once formed is not destroyed by acid, but simply rendered invisible. If a few drops of acid be added to a finely pigmented culture of any of the species employed, the color is completely discharged, but the addition of alkali brings it back as vividly as ever. On adding acid again and then alkali the same change again occurs, and this can be repeated for at least six times upon the same culture without any perceptible weakening of the intensity of the pigment. When, furthermore, a well-developed culture is made quite strongly acid with HCl and allowed to stand for twenty-four hours, the color reappears when alkali is added with the same intensity as before. The existence of an aphanochromatic substance, at one time conspicuously visible in the form of the fluorescent pigment, at another colorless and not apparent, is clearly demonstrated by these experiments. This substance, however, is not formed in acid solutions.

The influence of acid upon the pigment is beautifully shown in the fermentation tube where the pigment acts as an indicator. In 3 per cent. glucose-broth (prepared from broth freed of muscle-sugar by the method suggested by Theobald Smith[20]), *B. viridans* for the first four to five days after inoculation develops no pigment although the broth becomes very turbid. No color appears when alkali is added to the broth at this stage, conclusively showing that the presence of acid interferes with the production of the pigment or its aphanochromatic double and does not simply mask the presence of this substance. After eight or

[20] Journ. Expt. Med. **2**: 543. 1897.

nine days, however, pigment makes its appearance. In such a nutrient medium, then, there is a struggle between two of the vital activities of this organism, namely, its ability to produce acid and its power to form alkali. The latter "function" in this case ultimately gains the upper hand.

In 3 per cent. saccharose broth the course of events is exactly reversed. Saccharose is less easily fermented by this species and the result is that pigment is formed at the outset and a two days' growth is well colored. As the saccharose becomes converted into acid the color slowly fades out and at the end of four days it has vanished.

The pigment formed by all the species is at first, as has been shown by Thumm and others, a delicate robin's egg blue, but as the solution becomes more alkaline owing to the bacterial growth, the color changes to green, and in strongly alkaline solutions is a deep green tint, showing no fluorescence. The addition of alkali to a solution containing the blue pigment produces at once the same change as is wrought more slowly by the alkali formed by the bacteria.

SUMMARY.

The upshot of my experiments may be summarized under the following heads:

1. *The presence of both phosphorus and sulfur is essential to the formation of the fluorescent pigment.*

The effect of almost infinitesimal quantities of sulfate in the presence of phosphate compels us to accept with some reserve the statements made by authors as to the production of fluorescence in media devoid of sulfur. Thumm's curious statement[21] that *B. fl. albus*, while it produces fluorescence in a medium composed of 1 per cent. ammonium succinate, 0.1 per cent. potassium phosphate, 0.04 per cent. magnesium sulfate, 0.02 per cent. calcium chlorid, and also produces a feeble green fluorescence when the magnesium sulfate is omitted, produces no fluorescence at all if the calcium chlorid also be left out, is perhaps most easily

[21] *Op. cit.* 42–43.

explicable on the supposition that a small amount of sulfate was present as an impurity in the latter salt.

2. *The nature of the base associated with the phosphorus and sulfur is not important.*

Thumm observed, as I have done, that the omission of calcium chlorid alone from a nutrient solution containing potassium phosphate and magnesium sulfate exerts no perceptible effect on the production of pigment, but that the omission of the magnesium sulfate alone causes a marked diminution in pigment production; in my own experiments a total inhibition. From this he draws the singular conclusion that "so far as the formation of pigment is concerned, magnesium cannot be replaced by calcium." It is not necessary to point out the fallacy involved in such a deduction.

3. The conclusions that may be drawn regarding the dependence of the fluorescent "function" upon the molecular constitution of the ammonium salts may be best appreciated through an examination of the constitutional formulæ of the organic acids whose salts were employed. The list is arranged, as far as possible, in order of fluorescigenic value.

Asparagin,	$COOH.\ CH_2.\ CH.\ (NH_2).\ CONH_2.$
Succinic acid,	$COOH.\ CH_2.\ CH_2.\ COOH.$
Lactic acid,	$CH_3.\ CHOH.\ COOH.$
Citric acid,	$COOH.\ C(OH).\ (CH_2COOH)_2.$
Tartaric acid,	$COOH.\ CHOH.\ CHOH.\ COOH.$
Uric acid,	$NH.CO.NH.CO.\overset{\frown}{C}=\overset{\frown}{C}.NH.CO.NH.$
Acetic acid,	$CH_3.\ COOH.$
Oxalic acid,	$COOH.\ COOH.$
Formic acid,	$H.\ COOH.$

Lepierre,[22] who studied the behavior of a species closely related to if not identical with *B. fl. putridus*, draws from his work the far-reaching conclusions that the fluorescence is intimately bound up with: first, the bibasicity of the acid; second, the existence in the molecule of at least two groups of CH_2. Conclusions analogous to these cannot be deduced from my own

[22] Ann. de l'Inst. Pasteur 9:643.

work. The difference between acetic acid on the one hand and oxalic and formic acids on the other is certainly significant, but that neither the carboxyl (COOH) nor the methylene (CH_2) grouping is essential to pigment production is shown by the availability of urate. The difference between tartrate and succinate, as well as that between formate and acetate does, however, clearly indicate that, other things being equal, the presence of the methyl or methylene group is coincident with superior nutritive value and fluorescigenic power.

4. The presence of acid in the medium not merely conceals the existence of the substance to which the color is due, but interferes with those vital activities of the bacilli which, in an alkaline solution, lead to the production of that substance.

5. Diffuse daylight is unfavorable to pigment production.

6. If chemical substances that prove, when in certain proportions, favorable to growth and to the production of pigment be present in excess of a certain quantity, the production of pigment will be checked, although growth may be more abundant than before.

I may add, as an *obiter dictum*, that since the pigment is of no discoverable advantage to the organisms possessing the power of producing it, its production is probably purely incidental and not an essential vital act. The "fluorescigenic function," upon which some bacteriologists dwell, is in my opinion simply the expression of certain changes wrought by the organism upon the nutrient substratum in which it lives. When the substratum contains certain compounds, the metabolic activities of the organism adjust themselves to these conditions and the metabolic products differ correspondingly. It is purely a matter of accident and of no physiological significance that under certain conditions one of these metabolic products happens to be fluorescent.

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LIST OF FLUORESCENT BACTERIA.

1. Bacillus aquatilis fluorescens (Lustig). Diagnostik der Bakterien des Wassers 64. 1893.

2. B. butyri fluorescens (Lafar). Arch. f. Hyg. **13**: 1. 1891.

3. B. chromo-aromaticus (Galtier). Compt. Rend. **106**.

4. B. cyaneofluorescens (Zangemeister). Centralbl. f. Bakt. **18** : 34. 1895.

5. B. cyanogenus (Ehrenberg). Cf. Hueppe. Mitt. a. d. Kaiserl. Ges. **2** :—.

6. B. dentalis viridans (Miller). Die Mikroorganismen der Mundhöhle 316. [2d ed.]

7. B. erythrosporus (Eidam). Cf. Cohn and Miflet, Cohn's Beiträge **3** : 128.

8. B. fluorescens albus (Zimmermann). Die Bakterien unserer Trink- und Nutzwässer 18. Chemnitz, 1890.

9. B. fluorescens aureus (Zimmermann), *op. cit.* p. 14.

10. B. fluorescens capsulatus (Pottien). Zeitschr. f. Hyg. **22** : 140. 1896.

11. B. fluorescens convexus (Wright). Memoirs National Acad. Sciences **7** : —. 1895.

12. B. fluorescens crassus (Flügge). Die Mikroorganismen **2** : 294. [3d ed.]

13. B. fluorescens foliaceus (Wright), *loc. cit.*

14. B. fluorescens immobilis (Flügge), *loc. cit.*

15. B. fluorescens incognitus (Wright), *loc. cit.*

16. B. fluorescens liquefaciens (Flügge). Die Mikroorganismen 289. [2d ed.]

17. B. fluorescens longus (Zimmermann), *op. cit.* p. 20.

18. B. fluorescens mesentericus (Tartaroff). Die Dorpater Wasserbakterien. 1891.

19. B. fluorescens minutissimus (Unna and Tommasoli). Monatsch. f. prakt. Dermatol. **9**.

20. B. fluorescens mutabilis (Wright), *loc. cit.*

21. B. fluorescens nivalis (Schmolck). Centralbl. f. Bakt. **4** : 545. 1888.

22. B. fluorescens non-liquefaciens (Flügge). Die Mikroorganismen 293. [3d ed.]

23. B. fluorescens ovalis (Ravenel). Memoirs National Acad. Sciences **8** : —. 1896.

24. B. fluorescens putridus (Flügge). Die Mikroorganismen 288. [2d ed.]

25. B. fluorescens putridus colloides (Tartaroff), *loc. cit.*

26. B. fluorescens Schuylkilliensis (Wright), *loc. cit.*

27. B. fluorescens tenuis (Zimmermann), *op. cit.* p. 16.

28. B. fluorescens (Lepierre). Ann. de l'Institut Pasteur **9** : 643. 1895.

29. B. graveolens (Bordoni-Uffreduzzi). Fortschr. d. Med., 1886.

30. B. iris (Frick). Virchow's Archiv., **116** : 292. 1889.

31. B. leucæmiæ canis (Lucet). Baumgarten's Jahrb. **1891**: 319.

32. B. lupuliperda (Behrens) (cf. Lafar, Technical Mycology 166).

33. B. melochloros (Winkler and Schröter). Centralbl. f. Bakt. **9**: 700. 1891.

34. B. oogenes fluorescens (5 varieties) (Zörkendörfer). Arch. f. Hyg. **16**: 300. 1893.

35. B. proteus fluorescens (Jäger). Zeitschr. f. Hyg. **12**: 525. 1892.

36. B. pyocyaneus (Gessard). Thèse de Paris, 1882.

37. B. rugosus (Wright), *loc. cit.*

38. B. scissus (Frankland). Zeitschr. f. Hyg. **6**: 398. 1889.

39. B. smaragdinus-fœtidus (Reimann). Inaug. Diss., 1887.

40. B. striatus viridis (Ravenel), *loc. cit.*

41. B. virescens (Frick), *loc. cit.*

42. B. viridescens non-liquefaciens (Ravenel), *loc. cit.*

43. B. viridis (Lesage). Arch. de Physiol. **20**: 212. 1888

44. B. viridis pallescens (Frick), *loc. cit.*

45. B. viridans (Symmers). Brit. Med. Journ. **2**: 1252. 1891.

46. B. viscosus (Frankland), *op. cit.* p. 391.

47. Bacterium osteophilum (Billet). Bull. Scient. de la France et de la Belgique, 1890.

48. Diplococcus fluorescens fœtidus (Klamann). Allg. Med. Centralzeitung, **1887**: 1347.

49. Micrococcus fluorescens (Maggiora). Gior. Soc. Ital. d'Igiene **16**.

50. M. versicolor (Flügge). Die Mikroorganismen 177. [2d ed.]